PROCESS AND COMPOSITION FOR ASSAYING CARBOHYDRATES

FIELD OF THE INVENTION

The present disclosure relates to processes, reagents, and compositions for assaying carbohydrates and carbohydrate-containing compounds. In particular, the disclosure relates to processes, reagents, and compositions for assaying carbohydrates associated with a glycoprotein.

BACKGROUND

As the interest in carbohydrate compounds increases, methods for the separation and detection of these carbohydrates, and their monosaccharide components are needed. In particular, the separation and detection of carbohydrates that are associated with biological materials, such as glycoproteins, glycolipids, proteoglycans, and the like, will facilitate the understanding of these complicated molecules and the interaction with and importance to cells. In some applications, an understanding of the sequence of monosaccharides forming these oligo and polysaccharides associated with these molecules is also desired. This analysis may also include a determination of the relative amounts of each monosaccharide component making up the complement of carbohydrates that are associated with the biological material.

Detection and quantification of carbohydrates has been accomplished by various spectroscopic methods, including ultraviolet, UV/visible, nuclear magnetic, and mass spectroscopy. However, carbohydrates from certain sources, such as biological extracts may be present in low amounts and accompanied by many other components that can hamper efforts to selectively and quantifiably detect the carbohydrate components of interest. In addition, the detection of carbohydrates in low amounts or at low concentration by spectroscopic methods may be limited by the absence of strongly absorbing functional groups or structural features on these carbohydrates. Many carbohydrates have their maximal absorbance in the UV region from about 180 to about 190 nm, but have low molar absorptivities and or small extinction coefficients. These factors may complicate the

selective detection of components that are present in small amounts and or are present in a complicated mixture of other components. Samples obtained from biological sources often have both these attributes.

Complex mixtures may require varying levels of purification prior to detection in order to reduce the number or intensity of interfering components that obscure the desired analysis. Such interfering components compete with the desired analysis by giving false signals, being mistaken for signals of interest, or interfering with the quantification of certain components having the same or similar retention times as the component of interest, such as co-eluting in chromatographic methods of separation. Methods that are developed to decrease the level of purification required can enhance the diversity of samples available for analysis.

SUMMARY OF THE INVENTION

Processes, compositions, and reducing reagent systems are described for analyzing carbohydrates, and derivatives thereof, and for analyzing carbohydrate-containing compounds, and derivatives thereof. In some embodiments, processes, compositions, and reducing reagent systems for analyzing carbohydrates associated with samples obtained from biological sources, such as a sample of glycoproteins, and the like are described. In other embodiments, processes, compositions, and reducing reagent systems for preparing fluorescent derivatives of carbohydrates, such as derivatives of 9-aminopyrene-1,4,6-trisulfonic acid (APTS), such as APTS prepared in citric acid, 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS), 5-aminonaphthalene-2-sulfonate (ANA), 3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde (CBQCA), 4-aminobenzonitrile (ABN), 5-(2-aminoethylamino)naphthalene sulfonic acid (1,5-EDANS), and the like are described. In other embodiments, processes, compositions, and reducing reagent systems for analyzing carbohydrates using an electrophoretic medium, such as slab or gel electrophoresis, or an electrophoretic medium contained in a column, such as capillary electrophoresis are described. In other embodiments, processes, compositions, and reducing reagent systems for analyzing carbohydrates using a fluorescence detector, such as laser induced fluorescence, and the like are described.

In some aspects, the processes, compositions, and reducing reagent systems described herein for assaying carbohydrates may be used to determine the presence of one or more monosaccharides, oligosacchariodes, and or polysaccharides. In other aspects, the processes, compositions, and reducing reagent systems described herein for assaying carbohydrates may be used to determine the relative amount or concentration of a monosaccharide or a plurality of monosaccharides associated with carbohydrate or carbohydrate-containing compounds, such as glycoproteins, glycolipids, proteoglycans, and the like. It is understood that such concentrations of monosaccharides may be determined relative to a standard, such as an internal standard, relative to other monosaccharides in a plurality of monosaccharides, or relative to an impurity. In other aspects, the compositions described herein include one or more glycoproteins, glycolipids, and or proteoglycans. In other aspects, the compositions described herein include one or more monosaccharides, oligosaccharides, and polysaccharides released from one or more glycoproteins, glycolipids, and or proteoglycans.

In other aspects, the processes, compositions, and reducing reagent systems described herein for assaying carbohydrates include a derivatizing agent having a fluorophore,. In certain variations, the fluorophore-containing derivative is a covalent derivative of the monosaccharides, oligosaccharides, and or polysaccharides being analyzed, illustratively through an amino bond. In other aspects, the processes, compositions, and reducing reagent systems described herein for assaying carbohydrates include a reducing agent, such as an aluminum or boron reducing agent, including boranes, sodium borohydrides, lithium borohydrides, aluminum hydrides, and lithium aluminum hydride reducing agents, and the like, and an organic solvent, such as a polar organic solvent, or a polar aprotic organic solvent such as dimethylsulfoxide (DMSO).

The process for assaying monosaccharides can be utilized to analyze, for example, monosaccharides associated with a glycoprotein. The process may also be utilized to determine the relative concentrations of the monosaccharides associated with the glycoprotein. The processes may include incubating a carbohydrate-containing compound, such as a glycoprotein, or a carbohydrate

derivative with reagents to free the sugars. Such incubations may involve hydrolytic chemical reactions, other chemical reactions, and enzymatic reactions.

In other embodiments, the processes, compositions, and reducing reagent systems described herein are formatted as a kit for analyzing carbohydrates and carbohydrate-containing compounds.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a flow chart of an exemplary embodiment of a process for assaying monosaccharides, for example, monosaccharides associated with a glycoprotein, in accordance with the present disclosure.

FIG. 2 is an electropherogram of APTS labeled monosaccharides prepared using DMSO (Example 1) or THF (Example 2). Peaks: (1) *N*-acetyl galactosamine (GalNAc), (2) *N*-acetylmannosamine (ManNAc), (3) *N*-acetylglucosamine (GlcNAc), (4) mannose (Man), (5) glucose (Glc), (6) fucose (Fuc), (7) galactose (Gal), (*) interfering impurity peak.

DETAILED DESCRIPTION

Processes for separating, detecting, and quantifying carbohydrates and carbohydrate-containing compounds are described herein. These methods are applicable to mono-, oligo-, and polysaccharides, and derivatives thereof, including glycolipids, protein-associated carbohydrates, such as glycoproteins and proteoglycans, and the like.

As used herein, the term "carbohydrate" includes sugars, monosaccharides, oligosaccharides, polysaccharides, and derivatives thereof, as well as proteins that have sugars associated with them, such as glycoproteins, glycopeptides, and proteoglycans. Such derivatives of carbohydrates include naturally occurring and synthetic derivatives, such as derivatives that include components that can be detected and or measured by fluorescence-based techniques. It is understood that the processes described herein are suitable for reducing sugars, as well as non-reducing sugars, such as ketoses.

In one aspect, the carbohydrates or sugars that may be analyzed by the processes described herein are monosaccharides and include pentoses, hexoses, and heptoses, such as xylose, arabinose, ribose, fucose, rhamnose, glucose, galactose, mannose, and the like; and amino sugars such as *N*-acetylglucosamine, *N*-acetylgalactosamine, and the like. In another aspect, the carbohydrates or sugars that may be analyzed by the processes described herein are oligosaccharides or polysaccharides, and include maltotetraoses, dextrins, such as dextrin 15, and the like. It is appreciated that with regard to oligosaccharides or polysaccharides, alpha and beta anomeric isomers or alpha and beta sugar linkages may be assayed using the processes described herein. Further, other sugar containing molecules, such as proteinaceous carbohydrates, such as glycoproteins, and the like may also be assayed using the processes described herein.

In another aspect, the monosaccharides or sugars that may illustratively be analyzed by the processes described herein include those associated with glycoproteins, such as *N*-acetyl galactosamine (GalNAc), *N*-acetylmannosamine (ManNAc), *N*-acetylglucosamine, mannose (Man), glucose (Glc), fucose (Fuc), galactose (Gal), and the like, or oligo and polysaccharides that include these illustrative monosaccharides. It is appreciated that the oligosaccharides and polysaccharides formed from these illustrative carbohydrates may also be assayed by the processes described herein.

In another embodiment, the carbohydrates are derivatized prior to analysis using the processes described herein. Such derivatives are illustratively covalently linked derivatives, complexation derivatives, and the like. In one aspect, the derivatization includes the covalent attachment of the derivatizing agent or a fragment thereof. Such derivatization illustratively proceeds by a reductive amination of the carbohydrate. Such reductive amination reactions are generally described in Larock, "Comprehensive Organic Transformations, a guide to functional group preparations," VCH Publishers (1989), the disclosure of which is incorporated herein by reference.

In one aspect of the processes including a reductive amination of carbohydrates, the reducing agent is a boron or aluminum reducing agent. Boron

reducing agents include, but are not limited to, BH_3, $(CF_3CO_2)_2BH$, $NaBH_4$, $NaBH_3CH$, $(n\text{-}Bu_4N)BH_3CN$, $LiHBEt_3$, and the like, and other di- and tri-substituted borohydride and cyanoborohydride reducing agents. Aluminum reducing agents include, but are not limited to $i\text{-}Bu_2AlH$, $LiAlH_4$, $Mg(AlH_4)_2$, $NaH_2Al(OCH_2CH_2OCH_3)_2$, and the like. The use of chiral reducing agents capable of asymmetric induction during the reduction reaction, such as $NaHB(O_2CCHR^1NR^2COR^3)_3$, wherein one or more or R^1, R^2, or R^3 is a chiral fragment or forms a chiral center, are contemplated to fall within the scope of the processes described herein. In another aspect, the reducing agent is present in excess of the carbohydrate, illustratively in the range from about 1.1 equivalents to about 10 equivalents, and/or in the range from about 1.1 equivalents to about 1.5 equivalents.

In another aspect, one or more solvents or solvent systems is used to perform the reductive amination reactions described herein, and include but are not limited to dimethyl sulfoxide (DMSO), dimethyl formamide (DMF), acetic acid, trifluoroacetic acid, water, and methanol, and combinations thereof. Illustrative solvent concentrations for such amination reactions include concentrations in the range from about 100 pM to about 100 mM, and in the range from about 1 nM to about 1 μM. In one illustrative aspect, the solvent includes DMSO, alone or as a co-solvent with one or more other solvents described herein. The amount of DMSO included in the amination reaction is illustratively in the range from about 10 to about 67 percent, and/or in the range from about 20 to about 50 percent.

In another aspect, the reductive amination reaction is performed with an amine compound that includes a fluorophore, to prepare fluorescent derivatives of the carbohydrates to be analyzed. The fluorophore included in the amine compound is capable of fluorescing when it is coupled to at least one of the carbohydrates to be analyzed. In one variation, the fluorophore is used in an molar amount equivalent to the carbohydrate. In another variation, the fluorophore is used in excess, illustratively in the range from about 1.1 equivalents to about 50 equivalents, and/or in the range from about 10 equivalents to about 50 equivalents. Carbohydrates are illustratively conjugated with components that may allow for a

more efficient detection of the carbohydrates, such as fluorescent molecules, or fluorophores, including but not limited to 9-aminopyrene-1,4,6-trisulfonic acid (APTS), such as APTS prepared in citric acid, 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS), 5-aminonaphthalene-2-sulfonate (ANA), 3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde (CBQCA), 4-aminobenzonitrile (ABN), 5-(2-aminoethylamino)naphthalene sulfonic acid (1,5-EDANS), and the like.

In another aspect, the reductive amination reaction is performed with citric acid, illustratively having a concentration of about 0.9 M. It is appreciated that the inclusion of citric acid, or other equivalent buffering acid, during the reductive amination reaction may improve the overall efficiency of the derivatization with the selected fluorophore-containing derivatizing agent.

In another embodiment, at least one of the carbohydrates or derivatives thereof is separated from other substances or components, including other carbohydrates. Such a separation may be accomplished by using a liquid chromatographic technique, including but not limited to paper chromatography, thin layer chromatography, column chromatography, flash, chromatography, or high performance or high pressure liquid chromatography. In addition, separation may be accomplished by using a gas chromatographic technique, including but not limited to gas, gas-liquid, or capillary gas chromatography. Further, separation may be accomplished by using an electric field, such as in gel, slab, or capillary electrophoresis.

In one aspect, the carbohydrate or carbohydrates are separated by slab electrophoresis, gel electrophoresis, or capillary electrophoresis. Illustratively, capillary electrophoresis may be used as a method of separating carbohydrates using conventional equipment. It is appreciated that many methods of performing electrophoresis or of using electrophoretic media are equally applicable to the processes described herein. Suitable equipment for performing capillary electrophoresis is commercially available, such as the P/ACE series Capillary Electrophoresis Systems (Beckman Instruments, Inc., Fullerton, CA), and the like. It is understood that the use of other sources of equipment apart from those of

Beckman Instruments does not depart from the scope and or spirit of the invention described herein.

In the derivitization procedures described herein, the complexes or conjugates of the carbohydrates to be assayed may be charged or may be capable of carrying a charge under appropriately selected conditions. A charge on the carbohydrate or derivative thereof may allow the separation of the carbohydrate derivative from other components, including other carbohydrate derivatives by using an electric field, such as by electrophoresis as described herein.

In one aspect, electrophoretic separation of the carbohydrates of interest is accomplished when the carbohydrates themselves are charged, or when the charge is generated on the carbohydrates capable of carrying a charge by placing them in a suitable solution, such as a buffered solution having a predetermined pH. The electric field is such that the carbohydrates and other components placed in an electrophoretic medium and subjected to the electric field will migrate along the electric field gradient. It is understood that migration along such an electric field gradient is dependent upon the electrophoretic medium and the nature, such as the chemical composition, of each of the components. In the case of carbohydrates, the charge may be generated by complexing of vicinal hydroxyl groups with polyvalent anions such as borate, illustratively at a pH of about 9 or greater, or at a pH in the range from about 9 to about 10. Alternatively, charges may be generated on carbohydrates that include amino groups, including derivatized carbohydrates, by lowering the pH of a solution of such carbohydrates illustratively to about 4 or less, or about 2.5 or less, or about 2.2 or less, with a suitably selected buffer, such as a phosphate buffer and the like. Illustrative concentrations of borate are in the range from about 10 to about 1000 mM, or in the range from about 100 mM to about 300 mM, or illustratively at about 150 mM. Besides borate, other buffering systems may also be used, such as phosphate buffers having a pH of about 7, and having a concentration in the range from about 10 to about 1000 mM, or at about 50 mM to about 300 mM, or at about 135 mM, or at about 50 mM; acetate buffers having a pH of about 5, and having a concentration in the range from about 10 to about 1000 mM, or in the range from about 100 mM to about 300 mM, or illustratively at about

100 mM; and organic amine salts, such as 3-morpholinopropane sulfonic acid (MOPS) buffers having a pH of about 7, and having a concentration in the range from about 10 to about 100 mM, or illustratively at about 60 mM.

In another embodiment, at least one of the carbohydrates is detected and optionally quantitatively determined. In one aspect, the detection method includes laser induced fluorescence using conventional equipment. It is appreciated that many methods of detection are equally applicable for use in the processes described herein, including other methods involving fluorescence, or other methods involving laser induced fluorescence.

Detection is accomplished by any of a number of techniques capable of detecting molecules, illustratively carbohydrates or other molecules with multiple hydroxyl groups. In addition, detection techniques that are particular to carbohydrate detection are suitable for use in the various embodiments of the processes described herein. Illustratively, techniques that are based on the detection of molecules, in the form of carbohydrate derivatives, that fluoresce are suitable. Detection using laser induced fluorescence involving a laser to excite the derivatized carbohydrates (both monosaccharides and polysaccharides) and subsequently monitoring the emissions of the derivatized carbohydrate components may be used. Such laser excitation techniques include any suitable laser source, such as argon ion lasers, He/Cd lasers, and the like. It is appreciated that the excitation wavelength is advantageously selected depending upon the nature of the components to be detected, such as wavelengths where the molar absorptivity is high. In such aspects, components at very low concentrations may be detected. It is further appreciated that interfering emission from unreacted materials, such as the fluorophore precursor, may be minimized by suitable selection of the excitation wavelength where the signals from derivatized molecules predominate over the signal from unreacted or uncoupled starting material, or where the latter do not have a significant emission spectrum, such as for example He/Cd lasers at 325 nm and argon ion lasers at 488 nm. In this aspect, derivatives of fluorophores described herein may have high molar absorptivities while the starting materials do not.

Suitable equipment for performing detection by laser induced fluorescence is commercially available, such as P/ACE Series Capillary Electrophoresis Systems fitted with a laser induced fluorescence detector (Omnichrome, Chino, CA), and the like. Such detectors may be fitted with a variety of laser sources. It is understood that the selection of laser source is dependent upon the excitation wavelengths of the compounds of interest, as well as the excitation wavelengths of the components that may interfere with the analysis of the compounds of interest. For example, laser source may be an He/Cd laser at 325 nm, or an argon-ion laser at 488 nm. Suitable filters are optionally included to facilitate the detection of compounds of interest and minimize the interference of other components, such as an emission filter of about 520 nm used in conjunction with an argon-ion laser. Further, suitable detection systems are described in U.S. Patent No. 4,675,300, the disclosures of such detection systems are incorporated herein by reference. It is appreciated that such detectors may be adapted for use with electrophoresis instruments, including commercially available electrophoresis instruments. It is understood that the use of other sources of equipment apart from those of Omnichrome does not depart from the scope and or spirit of the invention described herein.

It is understood that suitable excitation wavelengths may be selected after taking a fluorescence spectrum of certain standard components, or certain components that are expected to be in the mixture to be analyzed. For example, the wavelength having the greatest absorption, such as the lambda max, may be selected for improved sensitivity of those components. Alternatively a different wavelength may be selected to provide improved detection of some components while minimizing the detection of other components. Such fluorescence spectra may also serve as a set of standards, and may be acquired or obtained using any available instrument capable of taking such a spectrum, including but not limited to commercial instruments such as the UV/visible spectra obtainable on a Beckman 7500 diode-array spectrophotometer, and the fluorescence spectra obtainable on a Perkin-Elmer LS50 Luminescence Spectrometer (Beaconsfield, Buckinghamshire, U.K.), and the like.

In another embodiment, the processes described herein include incubating a sample containing carbohydrates for analysis, such as a sample containing a glycoprotein or a derivative thereof, with various substances prior to or contemporaneous with derivatization, analysis, or detection of the carbohydrates. In one aspect, the processes include incubating the glycoprotein in an acid solution, such as a TFA solution, and the like, to produce an incubation product. In one variation, the glycoprotein may be incubated in a dilute solution of TFA, illustratively having a concentration of about 0.1 M TFA or less, optionally at an elevated temperature.

In another aspect, the processes described herein include incubating a sample containing carbohydrates for analysis, such as a sample containing a glycoprotein or derivative thereof, in a concentrated solution of TFA, optionally at an elevated temperature. Illustratively, a glycoprotein, or a derivative or a degradation product thereof, can be incubated with a concentrated TFA solution, having a concentration of about 4 M TFA or greater at an elevated temperature to produce another incubation product. It is appreciated that other intermediate concentrations of such TFA solutions, such as concentrations in the range from about 0.1 M to about 4 M TFA may be used in variations of the processes described herein. In addition, various temperatures may be selected as needed to ensure that the desired incubation products are formed.

In another aspect, the processes described herein include incubating a sample containing carbohydrates for analysis, such as a sample containing a glycoprotein or a derivative thereof, with an enzyme, including but not limited to *N*-acetylneuraminic aldolase, alpha amylase, and the like, to produce an incubation product. Illustratively, the incubation includes incubating a with *N*-acetylneuraminic aldolase, illustratively from *E. Coli*, at about room temperature in a phosphate buffer at a near-neutral pH, such as a pH in the range from about 6 to about 8.5, illustratively at a pH of about 7.5 to produce the incubation product.

In another aspect, the processes described herein include incubating a sample containing carbohydrates for analysis, such as a sample containing a glycoprotein or a derivative thereof, with ammonium carbonate, illustratively having

a concentration of about 25 mM, to produce an incubation product. In another aspect, the processes described herein include incubating a sample containing carbohydrates for analysis, such as a sample containing a glycoprotein or derivative or degradation product thereof, with acetic anhydride.

In one variation, a glycoprotein, or a derivative or a degradation product thereof, is subjected reductive amination, such as reductive amination with 9-aminopyrene-1,4,6-trisulfonic acid and sodium cyanoborohydride, at about 1 M, in dimethyl sulfoxide (DMSO), optionally at an elevated temperature, or another compound described herein for reductively aminating.

It is understood that the incubation processes described herein may be performed in series, to produce a first incubation product, which is then subjected to another incubation process, to form a second incubation product, and so forth. It is also understood that the incubation processes described herein may be performed contemporaneously in various combinations, such as incubation with both ammonium carbonate and acetic anhydride as described herein.

In one illustrative embodiment, a serial incubation process is described. The illustrative process includes incubating a sample containing carbohydrates for analysis, such as a glycoprotein, in a solution of TFA, illustratively of about 0.1 M at an elevated temperature, to produce a first incubation product. The process may also include incubating the first incubation product with *N*-acetylneuraminic aldolase from *E. Coli* at about room temperature in a phosphate buffer at a pH of about 7.5 to produce a second incubation product. The second incubation product may be incubated in a TFA solution, illustratively of about 4 M TFA at an elevated temperature, to produce a third incubation product. The process may also include incubating the third incubation product with an ammonium carbonate solution of about 25 mM, and acetic anhydride to produce a fourth incubation product. The process may also include incubating the fourth incubation product with 9-aminopyrene-1,4,6-trisulfonic acid, with citric acid at about 0.9 M, and sodium cyanoborohydride, at about 1 M, in dimethyl sulfoxide, optionally at an elevated temperature to produce a fifth incubation product.

Accordingly, the fifth incubation product can be assayed with the processes for separation, detection, and quantification described herein. For example, the fifth incubation product may be separated by capillary electrophoresis, and detected by laser induced fluorescence. This detection method may also involve a quantification and identification procedure that is used to identify the monosaccharide carbohydrates associated with a glycoprotein subjected to the series of incubation steps illustratively outlined herein. Included in this quantification is a determination of the quantity of each monosaccharide degradation product relative to an internal standard, impurity, or relative to each other in the cases where more than one monosaccharide degradation product is detected.

In one aspect, an internal standard is included in the sample prior to the derivatization procedure. In one variation, the internal standard is a carbohydrate that will not interfere with the analysis of the one or more carbohydrates originally present in the sample. For example, the carbohydrate may be lactose. An internal standard taking the form of a carbohydrate placed in the sample prior to derivitization may also be derivatized. In this variation, the internal standard is generally separated and detected along with the other carbohydrate or carbohydrates to be assayed. It is appreciated that using a non-interfering carbohydrate as an internal standard may also allow for the correction of standard or systematic errors affecting quantification of the sample components.

In another illustrative embodiment, a serial incubation process is described, as depicted in FIG. 1. The illustrative process includes incubating a glycoprotein having associated with it carbohydrates for analysis. In step (a), the glycoprotein sample is optionally dried prior to the process to allow for the absolute quantification of the carbohydrate, rather than the relative quantification of the mono, oligo, and polysaccharide components, and placed in a suitable reaction chamber, such as a SafeLock Eppendorf tube. In step (b), the sample is incubated with TFA, such as 4M TFA, illustratively at an elevated temperature of about 95 °C for about 2 h. In step (c), the sample is dried, illustratively in a Speed Vac Concentrator, re-suspended in water, and dried again in step (d). In step (e), the sample is incubated contemporaneously with ammonium carbonate, illustratively at

about 25 mM and at a pH of about 9.5, and with excess acetic anhydride, illustratively at a ratio of about 1000 g/g of glycoprotein. In step (f), the sample is dried, illustratively in a Speed Vac Concentrator. In step (g), an internal standard, illustratively lactose at a ratio of about 10^{-3} moles/g of glycoprotein, is introduced into the sample. In step (h), the sample is incubated with citric acid, and derivatized, illustratively by reductive amination using sodium cyanoborohydride and 9-aminopyrene-1,4,6-trisulfonic acid (APTS) at about 55 °C for about 2 h in DMSO/H_2O at a ratio of about 1:2. In step (i), the sample is diluted with water, illustratively by about 50-fold, and analyzed by Capillary Zone Electrophoresis (CZE), illustratively using bare fused silica capillary with anodic-electroosmotic flow, and detected by Laser-Induced Fluorescence (LIF).

It is appreciated that the processes described herein may be used for various analytical determinations, including but not limited to carbohydrate and glycoprotein analysis and sequencing, drug analysis, and other industrial sugar and carbohydrate analytical procedures, or diagnostic procedures, such as the analysis of various biologically derived samples.

In another embodiment, the processes described herein for assaying carbohydrates are equally applicable to isolated samples of carbohydrates, that have been optionally purified, as well as to impure samples, including crude extracts, of or from natural or biological sources, containing carbohydrates. In one aspect, an impure extract from a biological source is derivatived with a fluorophore. Derivatization conditions are selected to optimize the detection of carbohydrates and minimize the detection of other components of the mixture. It is appreciated that such selection of conditions also allows the use of an excess of the derivatizing agent to ensure maximal detection of the carbohydrates, while minimizing the interference from impurities or other component that are not of interest to the particular assay.

It is appreciated that the processes described herein are suitable for or may be adapted for the detection of very low concentrations of carbohydrates and derivatives thereof, such as concentrations in the micromole, nanomole, or picomole ranges of concentrations.

EXAMPLES

Example 1. Reductive amination in DMSO.

A solution of seven sugars ((1) GalNAc, (2) ManNAc, (3) GlcNAc, (4) Man, (5) Glc, (6) Fuc, (7) Gal) was admixed with lactose as an internal standard. Following the procedure described in FIG. 1, and in accordance with the invention described herein, the mixture was treated with APTS prepared in citric acid and sodium cyanoborohydride prepared in DMSO. Heating was performed in an oven. The resulting sample was analyzed by CZE-LIF, as described herein. The electropherogram is shown in FIG. 2, the trace labeled Example 1.

Example 2. Reductive amination in THF.

A solution of seven sugars ((1) GalNAc, (2) ManNAc, (3) GlcNAc, (4) Man, (5) Glc, (6) Fuc, (7) Gal) was admixed with lactose as an internal standard. Following the procedure described in FIG. 1, and in accordance with the invention described herein, the mixture was treated with APTS prepared in citric acid and sodium cyanoborohydride prepared in THF. Heating was performed in an oven. The resulting sample was analyzed by CZE-LIF, as described herein. The electropherogram is shown in FIG. 2, the trace labeled Example 2.

FIG. 2 shows that the reductive amination performed in THF (Example 2) leads to the formation of a impurity (* peak) that interferes with the complete analysis of the mixture of seven sugars, as compared to the reductive amination performed in DMSO (Example 1). The peak appearing at about 9.7 minutes in Example 2 is absent from Example 1. This interfering or impurity peak makes it difficult to identify the peak for the monosaccharide ManNAc, which, if present, may overlap the impurity peak. The peak at about 8.5 minutes in each example corresponds to the lactose internal standard.

Without being bound by theory, it is suggested that the additional peak seen by the detector may arise from incomplete reaction intermediates, or from by-products formed in these reactions. The inclusion of DMSO, or other polar solvent, in the reducing reagent system may effect more complete reaction to avoid

the detection of incomplete reaction intermediates, or may discourage the formation of by-products of the reactions. It should be appreciated that the presence of DMSO appears to suppress the formation of by-product peaks, or ghost peaks, which can interfere with the analysis of carbohydrates or mixtures of carbohydrates by capillary electrophoresis with laser induced fluorescence detection.

The foregoing description of illustrative and exemplary embodiments, and the variations and aspects thereof, are each intended to be illustrative of the invention claimed herein. However, this description should not be construed or interpreted as limiting the invention. Further, the accompanying Examples describing specific embodiments are intended to be illustrative of the invention described herein, and should not be construed or interpreted as limiting the invention. It is therefore understood that protection is desired for all changes and modifications that come within the scope and the spirit of the disclosure. For example, while the boron and aluminum reducing agents have been described and exemplified herein, it is to be understood that other reducing agents capable of converting the carbohydrates described herein into derivatives, such as aromatic amine derivatives, that may be detected by fluorescence include silane reagents such as Et_3SiH/CF_3CO_2H, and the like; iron reagents such as $NaFe(CO)_4H$, $KFe(CO)_4H$, and the like; and transition metal reagents, such as $LaNi_5H_6$, and the like.